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Enzymatic Saccharification of Woody Plants

I. Effects of Expanded Softening and Ball-milling on Enzymatic Saccharification*

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Abstract——Domestic hard wood mixtures were subjected to expanded softening (160–190°C, 6–15 kg/cm^2) followed by enzymatic saccharification. No substantial differences were observed in the chemical composition and degree of crystallinity, except for that of pentosan. Xylan portion is decomposed during expanded softening. Expanded softening did not enhance the accessibility of enzymes to substrates in contrast to the case of ball-milling, but appeared to inhibit saccharification. No enzyme preparations commercially available could digest more than 20%. Present results suggest that the condition of expanded softening is too mild to decompose lignin and that more drastic condition is necessary to achieve sufficient amount of saccharification.

1. Introduction

Since the accessibility of cellulolytic enzymes to cellulose in native wood is low, various physical, chemical and biological pretreatments have been reported to overcome this problem[1~10]. Of these three pretreatments physical pretreatment seems to be the most preferable because no necessity for using any organic solvents or reagents which causes somewhat laborious operations for their recovery and regeneration system. It has previously been reported that ball-milled-[11] and three roll-milled-[12] wood meals could be effectively digested with one to one mixture of enzyme preparations of Onozuka and Cellulosin. However, problems still remain for development of rapid and inexpensive pretreatment, since such ball- and roll-milling treatments have disadvantages on the following points: a) Ball-milling is time-consuming and expensive; b) Roll-milling needs disperse medium; and c) Roll-milling is not effective without aid of ball-milling. Recently, a technique of expanded softening was developed for utilization of rice hulls[13]. This technique has an outstanding characteristic in that, when the sample is compressed to 6–15 kg/cm^2, the temperature of the sample increases to 160–190°C. Asano *et al.* have shown the effective application of expanded softened rice hulls to animal feeding[14].

The aim of the present report was to investigate the effect of expanded softening on enzymatic saccharification of woody materials. Suitable applications of eleven

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commercially available enzyme preparations for saccharification of woody materials were also examined.

2. Experimental

2.1 Materials

Domestic hard wood mixtures containing about 30% of beech were supplied from Sanyo Kokusaku Co., Ltd. and milled to 60–80 mesh in a Willey Mill. Rice hull was supplied from Tsurumi soda, Co., Ltd. Pulp flock (bleached pulp for layon prepared from 7 to 3 mixture of mangrove and hard woods), fine pulp (unbleached fibers containing ray cells) and pulp refuse (trailing which remained on the knot-screen) were supplied from Kokoku Jinken Co., Ltd. Avicel SF was obtained from Asahikasei Kogyo, Co., Ltd. Extractive free pine and beech woods were ball-milled for 24 hr under N_2 using ceramic balls. Other enzyme substrates were the same as previously described[15]. Eleven commercially available enzyme preparations were listed in Table I.

Table I. Enzymes used for saccharification.

Enzymes		Origin
1. cellulosin AC	Ueda Kagaku Kogyo Co.	*Aspergillus niger*
2. Cellulase Onozuka R-10	Yakult Pharmaceuticals Co.	*Trichoderma viride*
3. Cellulase (M)	E. Merk, Darmstadt	*Oxiporus sp.*
4. Cellulase (S)	Serva Fine Chemicals Co.	*Aspergillus niger*
5. Meicelase CEPB-5042	Meijiseika Co.	*Trichoderma viride*
6. Cellulase 2000 CUN	Nagase Biochemicals Co.	*Aspergillus niger*
7. Cellulase T-AP-4	Amano Pharmaceuticals Co.	*Trichoderma viride*
8. Cellulase AP-3	Amano Pharmaceuticals Co.	*Aspergillus niger*
9. Dricelase	Kyowa Hakko Kogyo Co.	*Irpex lacteus*
10. Hemicellulase (KL)	Koch-Light Laboratories	Fungal (not known)
11. Hemicellulase (ICN)	ICN Pharmaceuticals	Fungal (not known)

2.2 General methods

Klason lignin and acid soluble lignin contents were determined by the TAPPI standard methods. The lignin content was also determined by the acetyl bromide method[16]. Holocellulose content was determined by the method of Uprichard[17]. Pentosan, α-cellulose, ash, cold and hot water extracts, 1% sodium hydroxide extract, moisture, and alcohol-benzene extract contents were determined by the JIS standard methods. The uronic acid anhydride content was determined by the semimicro-determination method of Johansson *et al.*[18]. Acetyl content was estimated by g.l.c. on a column (2 m × 0.3 cm) of tetramethylcyclobutanediol adipate-4% phosphoric

acid on Chromosorb W at 120°C using *n*-propionic acid as an internal standard[19]. The neutral sugar composition of samples was determined by g.l.c. on a column (2 m×0.3 cm) of 3% ECNSS-M on Gas Chrom Q at 180°C using methyl β-D-glucopyranoside as an internal standard[20], after hydrolysis according to Saeman *et al.*[21] G.l.c. was conducted on a Shimadzu GC-4CM gas chromatograph, equipped with flame-ionization detectors. Configurations of the monosaccharides were determined by g.l.c. on a S.C.O.T. column of SP-1000 at 200°C after conversion into acetylated (+)-2-octyl D- and L-glycosides[22]. Protein content was determined by the method of Lowry *et al.*[23] using bovine serum albumin as standard with no correction for differences in chromogenicity.

2.3 Enzyme assays

1) Assays for aryl Glycosidases.

The activities of nine aryl glycosidases (α-glucopyranosidase, β-glucopyranosidase, α-galactopyranosidase, β-galactopyranosidase α-mannopyranosidase, β-mannopyranosidase, α-xylopyranosidase, β-xylopyranosidase and α-arabinofuranosidase) were assayed by using appropriate *p*-nitrophenyl D-glycopyranosides and L-glycofuranoside as substrates. The standard reaction mixture contained 0.1 ml of enzyme solution, 0.1 ml of 0.5 M sodium acetate buffer (pH 4.6), 0.1 ml of 6.5 mM substrate solubilized in distilled water, and distilled water in a final volume of 1.0 ml[15]. After incubation for 10 min at 37°C on a Monod-shaker, the reaction was terminated by addition of 5.0 ml of 0.6 M sodium carbonate. The liberated *p*-nitrophenol was determined spectrophotometrically at 410 nm. One unit of the enzyme activity is defined as the amount of enzyme which liberates 1 μmole of *p*-nitrophenol per min under the condition described above.

2) Assays for cellulases, hemicellulases and other polysaccharases.

Cellulases were assayed by using Avicel SF and sodium salt of carboxymethylcellulose (CMC) as substrates. Hemicellulases were assayed by using 4-*O*-methylglucuronoxylan from beech, arabinoxylan from bagasse, arabinogalactan from larch, mannan from ivory nut, and arabinan from sugar beet as substrates for xylanase, arabinoxylanase, galactanase, mannanase, and arabanase, respectively. The other polysaccharases were assayed using amylose, pectin, and dextran for amylase, pectinase and dextranase activities, respectively. The standard reaction mixture contained 0.1 ml of enzyme solution, 0.25 ml of 1% substrate or 10 mg of ball-milled wood meal or a sheet of filter paper (1 cm×1 cm, Toyo Roshi No. 51, specifically prepared papers for cellulase)[24], 0.1 ml of 0.5 M sodium acetate buffer (pH 4.6), and distilled water in a final volume of 1.0 ml. 4-*O*-Methylglucuronoxylan and arabinoxylan were solubilized in aqueous 1 N sodium hydroxide solution, neutralized with 1 N

HCl and used for xylanase substrates. After incubation for 30 min at 40°C on a Monodshaker, the reducing sugar formed was determined by the dinitrosalicylic acid (DNS) method[25]. One unit of the enzyme which liberates 1 μmole of monosaccharide under the condition described above.

2.4 Expanded softening treatment

The apparatus for expanded softening was 'Press Pander' manufactured by Tsurumi soda Co., Ltd., Tsurumi, Yokohama. The schematic illustrations of Press Pander were shown in Figs. 1 and 2. This apparatus was firstly developed for utilization of rice hulls and was applied for the first time to woody materials. The domestic hard wood mixture and rice hull were subjected to expanded softening. It should be stressed that the temperature increased to 160–190°C without any external thermal supply, when the sample was compressed to 6–15 kg/cm^2 by the taper screw. Then, the compressed sample was cut at the end of the taper screw and gushed out

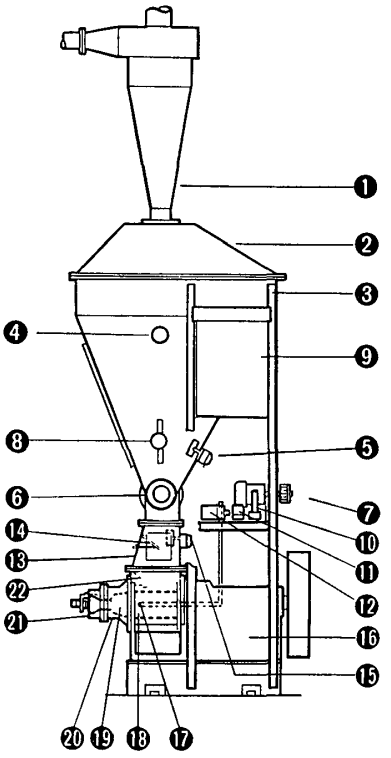


Fig. 1 Schematic illustration of Press Pander.
(1) Cyclonic selector; (2) Hopper; (3) Steel frame; (4) Upper Level switch; (5) Lower level switch; (6) Sample supplier; (7) Motor and flow adjuster; (8) Bridge breaker; (9) Water reservoir; (10) Flow valve; (11) Electromagnetic valve: (12) Water pump; (13) Inspection box; (14) Rectifier; (15) Emergency receiver; (16) Press pander; (17) Water nozzle; (18) Parallel screw; (19) Taper screw; (20) Presser vessel casing; (21) Eject nozzle; (22) Regulator.

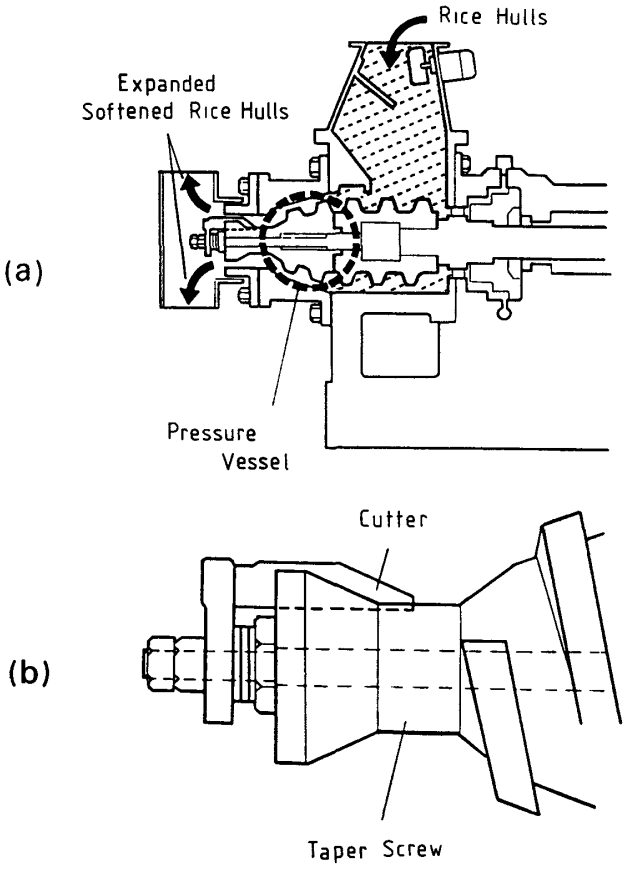
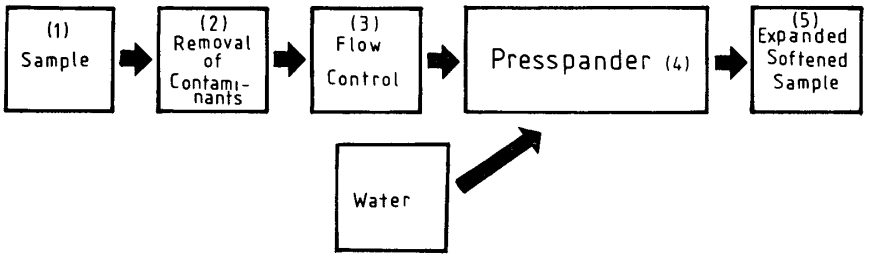


Fig. 2 Expanded illustrations of Press Pander and Taper screw.
(a); Expanded illustration of Press Pander.
(b); Expanded illustration of taper screw cutter.

through eject nozzle. The sample was turned into somewhat fluffy fibrous material by this treatment. The sample in the range of 60–80 mesh was used for further studies. The overall procedure is shown in the flow chart.



Flow chart of expanded softening

2.5 Enzymatic saccharification

Standard enzymatic saccharification was carried out at 40°C for 48 hr at substrate and enzyme concentrations of 1% and 0.1%, respectively. One hundred

mg of substrate and an appropriate amount of enzyme which corresponds to 10 mg of protein were inoculated in a L-type tube containing 10 ml of 0.1 M sodium acetate buffer. After a few drops of toluene were added as an antiseptic, each tube was shaken on a Monod-shaker. The sample remained after saccharification was recovered by filtration through a tared glass crucible (polosity 4) and weighed, and corrected for values obtained without enzymes. The reducing sugar content in the filtrate was determined by the DNS method. Time course of saccharification was determined at time intervals of 2, 4, 8, 12, 24 and 48 hr as described above. Dependence of enzyme concentration was determined as described above at substrate concentration of 1%, and enzyme concentrations of 0.025, 0.05, 0.1, 0.2, 0.35 and 0.5%, respectively.

2.6 Degree of crystallinity

Wood meal samples compressed into discs [20 mmϕ × 1 mm(T)] were used in this study. A Rigaku-denki Geigerflex B2021 equipped with a line focussing X-ray optical system and a Ni-filtered Cukα (35 KV, 20 mA) as a source of X-ray was applied. The measurement conditions were divergence slit 1°, scattering slit 1°, receiving slit 0.3 mm, time constant 2 sec, and scanning speed 1°/min. Two theta scannings were performed by the symmetrical reflection technique, and the X-ray diffraction intensity curves were obtained. After correction of the air scattering, these curves were separated into background, amorphous and crystalline regions according to the method of Jayme and Knolle[26]. The degree of crystallinity were computed according to the following equation:

$$(\text{D.C.}) = \frac{\int I_{cr}(2\theta)\, d(2\theta)}{\int I_{am}(2\theta)\, d(2\theta) + \int I_{cr}(2\theta)\, d(2\theta)}$$

where θ represents the Bragg angle, I_{am} and I_{cr} (2θ) are the scattering intensities from amorphous and crystalline regions at 2θ, respectively.

3. Results and Discussion

3.1 Effects of expanded softening on chemical composition

The chemical composition of the native domestic hard wood mixture was compared with that after expanded softening (Table II). No substantial differences were observed except the amounts of cold water, hot water, 1% sodium hydroxide extracts and α-cellulose contents. It is suggested that some water soluble components of the native wood may converted into water insoluble form. The result that the increase of α-cellulose content is almost equilivalent to the decrease of the amount of water soluble materials may indicate that water soluble components are immobilized

Table II. Summative chemical composition of the domestic hard wood mixtures.

Component	Native	Expanded softened
Moisture	17.3	1.8
Ash	0.8	0.9
Acetyl	2.2	1.6
Uronic anhydride	4.7	3.8
Pentosan	20.2	16.6
Holocellulose	74.9	75.8
α-Cellulose	37.4	44.9
Lignin		
Klason lignin	23.8	22.5
Acid-soluble lignin	2.0	1.8
Acetyl bromide	26.7	25.3
Alcohol-benzene extract	2.1	3.1
Hot water extract	9.5	3.2
Cold water extract	7.4	1.1
1% Sodium hydroxide extract	24.7	32.4

*Values in per cent of native and explosively softened wood meals.

on to the α-cellulose by condensation reaction, although the mechanism of this reaction is not clear now. The result of the increase in the content of 1% sodium hydroxide soluble materials and the decrease in the pentosan content indicates that hemicellulose such as xylan is somewhat decomposed by expanded softening, since pentopyranoside linkages are considerably weaker than the hexopyranoside linkages. In order to confirm this postulation, neutral sugar composition was analyzed before and after expanded softening. The results are shown in Table III which includes the results of rice hulls as comparison. It is clear that substantial amount of xylose residues

Table III. Neutral sugar composition of the domestic hard mixtures and rice hulls*.

	Domestic hard wood mixture		Rice hull	
	Native	Expanded softened	Native	Expanded softened
L-Rhamnose	0.9	0.9	0.4	0.3
L-Arabinose	2.2	1.8	4.0	3.7
L-Xylose	35.6	30.0	45.9	38.1
D-Mannose	0.5	0.8	0.3	0.8
D-Galactose	2.1	1.7	2.4	2.0
D-Glucose	58.7	64.8	46.9	55.0

*Values in per cent of neutral sugars.

was lost after expanded softening in contrast to the increase in glucose content. Wood and rice hull represent similar differences in neutral sugar composition. On the basis of the results presented above, it is concluded that pentosan, particularly, xylan portion of wood is partially decomposed during expanded softening. The results obtained for the crystallinity of cellulose are given in Table IV. It is clear that the expanded softening used here did not give rise to decrystallization of cellulose in wood and that ball-milling procedure caused marked decrease in crystallinity of cellulose confirming the previous results[27~29]. It is probable that lignin portion was not seriously affected by expanded softening, since lignin and alcohol-benzene extract contents did not increased after this treatment. Further studies are necessary to examine more effective expanded softening to affect lignin component.

Table IV. Degree of crystallinity (D.C.) of the substrates and cellulosic materials.

Component	D. C. (%)
Ball-milled pine wood meal	13.4
Ball-milled beech wood meal	9.8
Domestic hard mixture	
Native	50.1
Expanded softened	49.4
Pulp flock	57.9
Fine pulp	61.6
Pulp refuse	58.8
Whatman CF-II cellulose	76.6
Avicel SF	66.7

Table V. Activities of aryl glycosidases*.

Enzymes	α-Glc**	β-Glc	α-Ga l	β-Gal	α-Man	β-Man	α-Xyl	β-Xyl	α-Ara
Cellulosin AC	2,2	79.1	77.6	54.2	0.5	19.15	7.1	38.0	18.5
Onozuka R-10	0.9	93.3	57.4	0.4	0.0	0.1	0,4	3.9	21.7
Cellulase (M)	1.7	30.0	1.4	1.0	0.1	0.1	0.1	8.5	29.1
Cellulase (S)	1.1	102.1	26.7	83.6	0.2	5.1	1.4	31.4	13.5
Meicelase	0.1	73.4	0.8	0.1	0.0	0.0	0.0	0.7	4.3
Cellulase 2000 CUN	1.8	157.5	160.3	43.0	0.2	102.8	1.3	18.0	21.3
Cellulase T-AP-4	0.4	45.5	20.6	10.1	0.2	10.2	0.4	1.3	2.9
Cellulase AP-3	1.4	49.2	23.3	12.3	0.0	18.3	0.2	0.5	0.2
Dricelase	1.1	172.5	25.1	14.3	0.6	0.6	1.3	4.7	22.9
Hemicellulase (KL)	0.0	16.3	7.2	0.7	1.2	0.0	0.0	6.6	0.0
Hemicellulase (ICN)	0.0	25.5	1.2	0.6	0.	0.3	0.0	0.6	8.0

*Values in Units per mg of protein. **α-Glc=α-glucosidase, *etc.*

3.2 Selection of enzyme for saccharification

In order to select the enzyme which is suitable for enzymatic saccharification of woody plants, 11 commercially available cellulase and hemicellulase preparations were assayed for activities of aryl glycosidases and polysaccharases. The results are summarized in Tables V and VI. All enzyme preparations from *Aspergillus niger* have remarkably higher aryl glycosidase activities than those *Trichoderma viride* and other Basidiomycetes with the exception of α-glucosidase activity. Beta-glucosidase activities in the enzyme preparations from *Trichoderma viride* (Onozuka R-10 and Meicelase) are, however, as high as in the case of *Aspergillus niger*. Since β-glucosidase

Table VI-(I). Activites of Cellulases, hemicellulases and the other polysaccharases*.

Enzymes	CMCase	Avicelase	Xylanase	Arabinoxylanase	Galactanase	Mannanase	Arabanase
Cellulosin AC	27.3	1.7	47.7	35.2	1.5	28.1	17.4
Onozuka R-10	26.4	8.3	46.2	34.7	0.8	14.0	5.0
Cellulase (M)	25.4	3.7	36.5	27.1	0.3	20.2	4.1
Cellulase (S)	24.9	3.8	23.4	7.3	6.4	12.1	16.1
Meicelase	23.7	11.5	29.8	25.9	2.7	4.9	2.9
Cellulase 2000 CUN	23.5	1.8	18.8	2.9	1.4	31.2	8.1
Cellulase T-AP-4	14.4	4.2	57.3	40.0	2.8	12.2	17.3
Cellulase AP-3	30.5	2.2	50.4	70.1	11.0	24.0	17.6
Dricelase	21.6	2.6	58.7	35.4	1.3	15.0	8.2
Hemicellulase (KL)	10.0	5.7	79.7	21.7	66.8	134.5	95.1
Hemicellulase (ICN)	9.0	5.0	67.8	36.3	50.0	126.0	93.2

*Values in Units per mg of protein.

Table VI-(II). Activities of Cellulases, hemicellulases and the polysaccharases*.

	Amylase	Pectinase	Dextranase	Ball-milled pine wood	Ball-milled beech wood	Filter paper	Protein** content
Cellulocin AC	34.9	47.4	0.4	9.4	4.5	0.0	81.0
Onozuka R-10	20.6	28.8	0.2	12.8	7.6	4.5	55.6
Cellulase (M)	13.5	9.8	0.4	10.0	5.7	3.6	49.6
Cellulase (S)	36.4	30.2	0.2	9.3	1.5	0.0	52.8
Meicelase	3.8	0.7	0.0	18.7	21.2	10.0	83.2
Cellulase 2000 CUN	30.1	68.0	0.0	12.0	9.0	0.1	30.8
Cellulase T-AP-4	27.2	52.2	1.3	6.0	16.0	3.8	31.8
Cellulase AP-3	63.4	95.0	2.4	10.2	11.4	2.6	28.2
Dricelase	11.1	3.2	0.1	15.0	14.7	0.1	35.4
Hemicellulase (KL)	44.8	6.8	7.5	8.6	6.7	0.8	4.1
Hemicellulase (ICN)	84.0	14.7	4.5	7.3	7.0	1.9	4.8

*Values in Units per mg of protein. **Values in weight per cent of original samples.

is responsible for the saccharification of cellulose in wood, the enzyme preparations from *Aspergillus niger* and *Trichoderma viride* are applicable to saccharification of woody plants.

As for cellulase and the other polysaccharase activities, enzyme preparations from *Trichoderma viride* were different from those from *Aspergillus niger* and other Basidiomycetes. Enzymes from *Trichoderma viride* had higher Avicellase and filter paper degrading activities than those from the other enzymes confirming the results previously reported[6,30]. Since this enzyme is thought to be C_1 enzyme which attacks crystalline region of cellulose[31], enzyme preparations from *Trichoderma viride* may be suitable for saccharification of woody plants and cellulose. In contrast, enzyme preparations from *Aspergillus niger* had relatively higher degrading activities for CMC, hemicelluloses, amylose, pectin and dextran. Since CMCase plays an important role in saccharification of noncrystalline cellulose[31,32], and wood is composed of complex polysaccharides comprising cellulose, hemicellulose, pectin and a small amount of starchy polysaccharides, the combined use of enzyme preparations from *Trichoderma viride* and *Aspergillus niger* may be preferable for saccharification of woody plants. This was already verified by using Onozuka R-10 and Cellulosin AC or AP as one to one mixture for saccharification of ball-milled pine wood[11,12]. Present results further indicate that single enzyme preparation of Meicelase from *Trichoderma viride* have saccharifying activity higher than the one to one mixture of Onozuka R-10 and Cellulosin AC or AP as described later.

In the present experiments, we further used ball-milled pine and beech woods as enzyme substrates, since these wood meals have been shown to be effectively attacked by the enzyme without removal of lignin and closely similar to the native woods, and thus these wood meals may be the most suitable substrates for wood saccharification used in the present experiments. The results in Table VI showed Onozuka R-10, Meicelase and Driselase had almost equivalent enzyme activities. This result suggests that all these enzymes had similar saccharifying abilities against woody plants. The results in the later sections of this paper, however, clearly indicate that Meicelase is the most preferable for enzymatic saccharification of woody materials. Since Meicelase is peculior to the other enzymes only in the degradation ability of filter paper, it is postulated that the limiting factor of saccharification of woody plants is the activity of C_1 enzyme toward highly crystalline cellulose fibrils.

The specific activities of aryl glycosidases and polysaccharases in hemicellulose preparations were considerably high. However, because of smallness of the protein contents in these enzyme preparations, these enzymes are not adequate for the purpose of saccharification of woody plants.

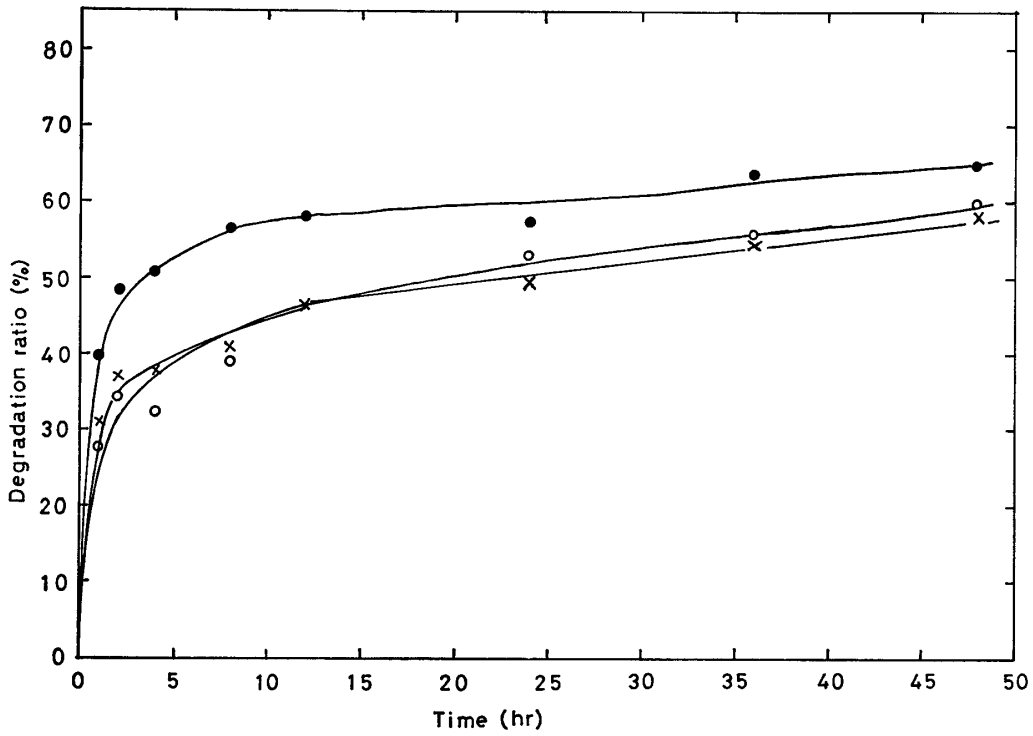


Fig. 3 Time course of saccharification.
●: Ball-milled pine wood meal. ○: Ball-milled beech wood meal. ×: Pulp flock.

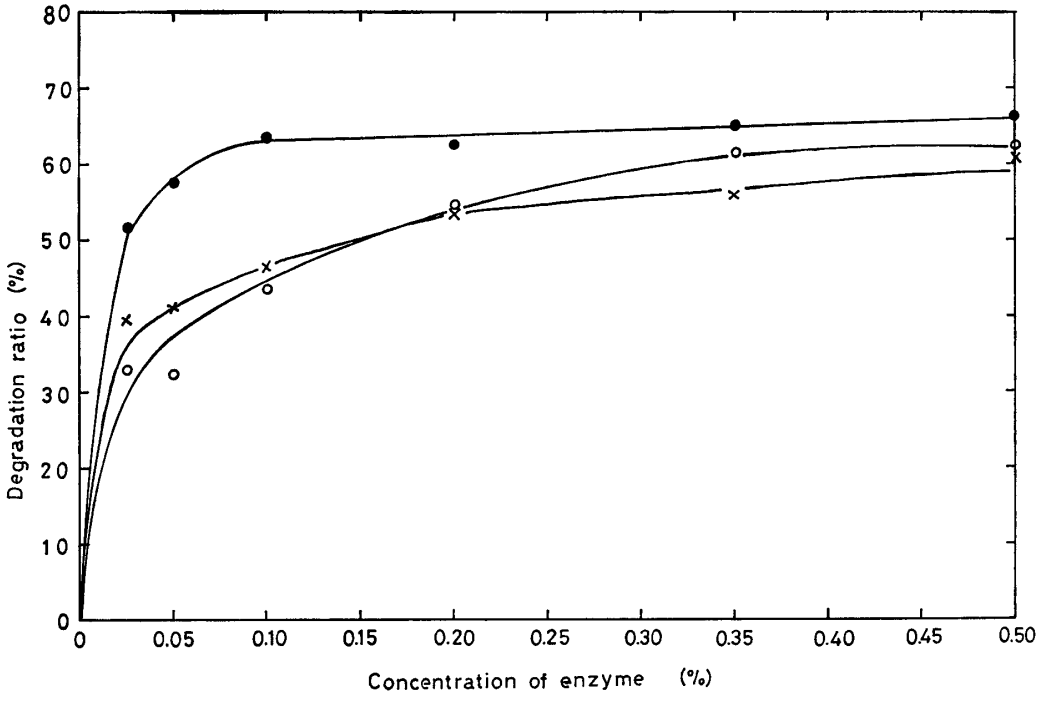


Fig. 4 Effect of enzyme concentration on saccharification.
●: Ball-milled pine wood meal. ○: Ball-milled beech wood meal. ×: pulp flock.

3.3 Time course of saccharification and effect of enzyme concentration on saccharification

Since preliminary experiments showed the marked superiority of Meicelase from *Trichoderma viride* for wood saccharification, we used this enzyme for the analyses of the time course of saccharification and the effect of enzyme concentration on saccharification. The results were shown in Figs. 3 and 4, using ball-milled pine and beech woods, and pulp flock as substrates. The degree of saccharification rapidly increased within 12 hr and enzyme concentration of 0.1%. After 12 hr, degree of saccharification slightly increased up to 48 hr. Prolonged incubation and high concentration of enzyme are not always necessary to achieve good results. On the basis of these results, we decided to use 0.1% for enzyme concentration and 48 hr for incubation time, respectively, in order to achieve maximal degree of degradation within a reasonable time and enzyme concentration.

3.4 Effect of expanded softening on enzymatic saccharification

At first, the various cellulosic materials and ball-milled pine and beech wood meals were subjected to enzymatic saccharification, using eleven different enzyme preparations. The results were listed in Table VII. As expected from the activity of each enzyme preparation, the enzymes from *Trichoderma viride* showed higher saccharifying ability than those from *Aspergillus niger* and the other Basidiomycetes. Meicelase has the highest saccharifying ability of the enzymes from *Trichoderma viride,* and degraded ball-milled pine wood meal (~65%) higher than did the one to one mixture of Onozuka R-10 and Cellulosin AC or AP (~58%)[11]. On the basis of the present results, it is concluded that Meicelase should be used for saccharification of woody materials. Although the activity of this enzyme preparations is high, the enzymes having the higher cellulose activities must be sought in order to minimize the time and amount of enzyme for saccharification.

Degree of saccharification of native domestic hard wood and rice hull were compared with those after expanded softening. The results were also shown in Table VII. Since this treatment enhances the hydrophilicity of rice hulls and 4 fold amount of water can be absorbed in expanded softened rice hulls[13], this treatment is expected to enhance the accessibility of enzyme toward substrate. The results, however, indicate that expanded softening did not enhance the accessibility of enzyme to substrates but appears to inhibit saccharification, in comparison with the degrees of saccharification of ball-milled pine and beech wood meals and various cellulosic materials. No enzyme preparations could degrade more than 23% of the hard wood mixtures. Rice hull seems to be more resistant to enzymic attack than wood. These results are not satisfied with the expected effects of expanded softening of woods

Table VII. Selection of enzyme for saccharification and effect of expanded softening of saccharification*.

	Domestic hard wood mixture				Ball-milled		Ball-milled		Pulp		Fine		Pulp	
	Native		Expanded softend		pine wood		beech wood		flock		pulp		refuse	
Cellulosin AC	5.0	(5.7)**	4.8	(5.4)**	15.1	(18.8)**	8.1	(10.0)**	2.1	(3.2)**	—	(2.0)*	1.6	(2.0)**
Onozuka R-10	7.9	(12.2)	9.9	(11.4)	57.2	(56.2)	52.6	(56.8)	21.9	(22.9)	30.3	(40.0)	30.3	(35.0)
Cellulosin AC + Onozuka R-10 (1 : 1 mixture)	12.1	(13.1)	12.7	(11.7)										
Cellulase (M)	14.6	(18.9)	19.6	(20.6)	46.7	(52.6)	55.9	(60.5)	15.7	(17.6)	20.0	(27.2)	25.1	(28.8)
Cellulase (S)	11.4	(20.6)	12.5	(20.2)	13.9	(18.0)	39.9	(42.2)	11.5	(15.0)	1.1	(2.5)	6.7	(7.5)
Meicelase	13.6 [13.4]	(20.0)	16.1 [11.5]	(20.9)	64.9	(67.8)	58.1	(61.5)	59.0	(86.9)	57.0	(65.0)	59.0	(62.5)
Cellulase 2000 CUN	1.3	(N.D.)	1.4	(N.D.)	11.3	(15.0)	14.8	(16.0)	—	(N.D.)	1.3	(N.D.)	7.1	(11.2)
Cellulase T-AP-4	11.8	(12.1)	12.4	(18.5)	56.3	(60.4)	57 5	(60.0)	51.2	(51.0)	26.6	(28.7)	33.0	(32.2)
Cellulase AP-3	10.8	(9.0)	13.1	(14.1)	40.2	(41.8)	21.7	(22.1)	9.7	(10.1)	6.3	(7.7)	6.5	(6.8)
Dricelase	2.1	(2.9)	4.6	(3.3)	31.7	(33.9)	42.0	(47.4)	—	(N.D.)	4.2	(4.7)	11.2	(11.7)
Hemicellulase (KL)	18.9	(22.8)	17.7	(21.7)	12.2	(10.5)	11.8	(19.0)	8.0	(15.0)	5.3	(5.8)	7.1	(7.9)
Hemicellulase (ICN)	6.2	(19.9)	15.7	(18.9)	5.9	(6.7)	10.1	(15.0)	26.8	(23.4)	2.9	(4.3)	6.4	(6.8)

*Values in percent weight loss after enzymatic treatment.
**Values in parentheses are per cent reducing sugar contents produced after enzymatic treatment.
***Values in square parentheses are per cent weight loss of rice hull after enzymatic treatment.
N.D.: not determined.

especially in rice hull. This may be ascribed to the possibility that the polysaccharides in wood and rice hull are still covered with lignin which inhibits the enzyme access toward the substrates of saccharides. The conditions of the present expanded softening seem to be too mild to decompose lignin, lignin-carbohydrate linkages and even polysaccharides. In conclusion, since the satisfactory results were not obtained, it is desirable to use more drastic conditions for achievement of saccharification effects which were reported for the explosion methods[33~37]. Then, expanded softening may thus be preferentially used for production of animal feeding, fertilizer, soil reforming agent, soil for seedling, compost, and culture for microorganisms including mushrooms.

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